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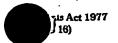
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NEWPORT

Cardiff Road Newport South Wales NP10 8QQ

Your reference

P32365/LMC/GST

2. Patent application number (The Patent Office will fill in this part)

0228139.2

n3 DEC 2002

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Dr Olga Kozlova-Zwinderman 239/5 Gilmerton Road Edinburgh **EH16 5TH** 

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

08519084001

Title of the invention

"Fungal Biosensor for Contaminant Detection"

Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

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Scotland House 165-169 Scotland Street

Glasgow G5 8PL

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1198013

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2 December 2002

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"Fungal Biosensor" 1 2 The present invention provides a method of using 3 fungus for determining the presence of at least one 4 toxic substance in a sample and for assisting in the 5 identification of the toxicant(s). 6 specifically there is provided a toxicity assay for 7 use in determining the presence of toxins and in 8 particular heavy metals and organophenols. 10 The release of contaminating substances into an 11 environment such as a waterway or an area of 12 agricultural land can have serious effects on the 13 ecosystems found in that environment. 14 important to be able to analyse these effects both 15 prior to the release of such contaminants so as to 16 manage their treatment or release, and after release 17 so as to determine and counteract their effects. 18 19 Current methods used to monitor water quality and 20 screen effluent generally involve chemical toxicity 21 However, these tests require a general idea 22

of the type of contaminant being tested for and can 1 2 be very expensive. 3 Biosensors are also used for toxicity testing and 4 are well known in the field. Toxicity depends on a 5 variety of factors including pH, temperature, 6 salinity and contaminant concentration, but depends 7 especially on the test organism used in the sensor. 8 9 One of the most commonly used organisms is the 10 bioluminescent bacterium, Vibrio fischeri. 11 bioluminescence involved is mediated by the 12 luciferin-luciferase enzyme system wherein light 13 emission is dependent on the electron transfer 14 chain. Any disruption to the electron transfer 15 chain, for example on exposure to a toxicant, 16 affects light emission. Measurement of changes in 17 light emission is therefore indicative of the 18 presence of a toxic substance. 19 20 This system, however, only provides a simple 21 indication of whether a contaminant is toxic or not. 22 No detailed information is obtained on how toxic the 23 contaminant is, nor is the contaminant identified. 24 25 The term toxicant as herein described relates to 26 compounds which are toxic to fungus and also to any 27 substance or compound with anti-fungal activity. 28 29 According to the present invention there is provided 30

1	an assay for use in determining the presence of a
2	known toxicant in a test sample, the assay
3	comprising the steps of;
4	- exposing a fungi transformed with a
5	recombinant aequorin gene to a test sample of
6	a substance,
7	
8	- measuring the luminescence produced by the
9	fungi,
10	
11	- converting the luminescence data into a
12	cytosolic free calcium ion concentration
13	trace,
14	
15	- and comparing at least one parameter of the
16	cytosolic free calcium ion concentration
17	trace with a bank of known toxicity reference
18	data.
19	
20	Preferably the cytosolic free calcium ion trace is a
21	plot of the cytosolic free calcium ion concentration
22	against time.
23	
24	Preferably the fungi transformed with a recombinant
25	aequorin gene is a filamentous fungi.
26	
27	More preferably the fungi is of the Aspergillus
28	species.
29	
30	Most preferably the fungi is Aspergillus awamori.
31	

Most preferably still the strain of Aspergillus 1 awamori is strain 66A. 2 3 Preferably the substance is a contaminant. 4 5 Preferably the substance is a contaminated sample. 6 7 Preferably the parameter is at least one or more 8 selected from the group comprising; lag time, rise 9 time, amplitude, full width half maximum, number of 10 cytosolic free calcium ion concentration increases, 11 percentage increase in final cytosolic free calcium 12 ion concentration resting level, and percentage 13 increase in recovery time. 14 15 In a further embodiment of the invention the test 16 sample is added in advance of the application of a 17 stimulus to the test sample. 18 19 Preferably the stimulus is at least one or more from 20 the group comprising; mechanical perturbation, hypo-21 osmotic shock, and change in external calcium 22 chloride concentration. 23 24 Preferably the test sample is added 1 minute to 1 25 hour prior to the application of the stimulus. 26 27 More preferably the test sample is added 5 minutes 28 prior to the application of the stimulus. 29 30 More preferably the test sample is added 30 minutes 31 prior to the application of the stimulus. 32

1	
2	In such instances, the parameters may include at
3	least one or more selected from the group
4	comprising; lag time, rise time, amplitude, full
5	width half maximum, number of cytosolic free calcium
6	ion concentration increase, percentage increase in
7	final cytosolic free calcium ion concentration
8	resting level, percentage increase in recovery time,
9	and percentage increase in pre-stimulating cytosolic
10	free calcium ion concentration resting level.
11	
12	Preferably luminescence is measured for between 1
13	minute and 5 hours following the application of the
14	stimulus.
15	
16	More preferably luminescence is measured for 5
17	minutes following the application of the stimulus.
18	•
19	A further embodiment of the present invention
20	provides a method of determining the amount of a
21	contaminant present in a test sample comprising the
22	steps of;
23	
24	- exposing a strain of fungi transformed with a
25	recombinant aequorin gene to a sample,
26	
27	- measuring the luminescence produced by the
28	fungi,
29	
30	- converting the luminescence data into a
31	cytosolic free calcium ion concentration
32	trace,

· )

1	
2	<ul> <li>and comparing at least one parameter of the</li> </ul>
3	cytosolic free calcium ion concentration
4	trace with a bank of pre-prepared toxicity
5	reference data.
6	
7	Preferably the cytosolic free calcium ion trace is a
8	plot of the cytosolic free calcium ion concentration
9	against time.
10	
11	Preferably the fungi transformed with a recombinant
12	aequorin gene is a filamentous fungi.
13	
14	More preferably the fungi is of the Aspergillus
15	species.
16	<u>.                                    </u>
17	Most preferably the fungi is Aspergillus awamori.
18	
19	Most preferably still the strain of Aspergillus
20	awamori is strain 66A.
21	
22	Preferably the substance is a contaminant.
23	
24	Preferably the substance is a contaminated sample.
25	•
26	Preferably the parameter is at least one or more
27	selected from the group comprising; lag time, rise
28	time, amplitude, full width half maximum, number of
29	cytosolic free calcium ion concentration increases,
30	percentage increase in final cytosolic free calcium
31	ion concentration resting level, and percentage
22	increase in recovery time.

1 In a further embodiment of the invention the test 2 sample is added in advance of the application of a 3 stimulus. 4 5 6 Preferably the stimulus is at least one or more from 7 the group comprising; mechanical perturbation, hypoosmotic shock, and change in external calcium 8 chloride concentration. 9 10 Preferably the test sample is added 1 minute to 1 11 hour prior to the application of the stimulus. 12 13 More preferably the test sample is added 5 minutes 14 prior to the application of the stimulus. 15 16 17 More preferably the test sample is added 30 minutes prior to the application of the stimulus. 18 19 In such instances, the parameters may include at 20 least one or more selected from the group 21 comprising; lag time, rise time, amplitude, full 22 width half maximum, number of cytosolic free calcium 23 ion concentration increase, percentage increase in 24 final cytosolic free calcium ion concentration 25 resting level, percentage increase in recovery time, 26 27 and percentage increase in pre-stimulating cytosolic free calcium ion concentration resting level. 28 29 Preferably luminescence is measured for between 1 30 minute and 5 hours following the application of the 31

32 stimulus.

1 More preferably luminescence is measured for 5 2 minutes following the application of the stimulus. 3 4 A first experiment comprises testing the effect of 5 pre-incubation of Aspergillus awamori with toxicants 6 on cytosolic free calcium ion concentration response 7 to an increase in external calcium chloride. 8 9 A further set of experiments described herein shows 10 attempts to obtain characteristic data for a range 11 of different toxicants at a number of different 12 The results demonstrate that each concentrations. 13 toxicant at each concentration produces a 14 distinctive cytosolic free calcium ion concentration 15 trace whose traits could be used to identify and 16 characterise a toxicant present in a test sample. 17 18 A final experiment attempts to determine whether it 19 is possible to identify and characterise individual 20 toxicants from testing samples of mixtures of 21 toxicants in different proportions. The traces . 22 produced are distinct for each mixture. 23 24 These results show that it is possible to 25 characterise and identify a specific toxicant from a 26 test sample by using the characteristic data 27 obtained from a cytosolic free calcium ion 28 concentration trace. 29 30 The parameters referred to herein relate to the 31 following; 32

1 Lag Time, the time from addition of the test sample 2 to the time when the cytosolic free calcium ion 3 concentration, [Ca2+]c, began to rise; 4 5 Rise Time, the time from addition of the test sample 6 to the time at which maximum [Ca2+]c was reached; 7 8 Amplitude, the maximum [Ca2+] c reached during the 9 experiment; 10 11 Full Width Half Maximum, the width of the transient 12 at the point where the amplitude equals half of the 13 maximum amplitude of the transient; 14 15 Number of [Ca<sup>2+</sup>]<sub>c</sub> Rises, the number of peaks in 16  $[Ca^{2+}]_{c}$ 17 18 Percentage Increase in Final [Ca2+] c Resting Level, 19 the percentage increase in resting [Ca2+] c at the end 20 of the experiment, where the control value is taken 21 to be 100%; 22 23 Percentage Increase in Recovery Time, percentage 24 increase in recovery time where recovery time 25 represents the total amount of [Ca2+]c released 26 during the period of time from the point when the 27 maximum amplitude following calcium chloride 28 treatment was achieved to the point when the [Ca2+]c 29 reached its final resting level. Recovery time was 30 initially calculated for control cultures. 31 control this period of time was calculated as 250 32

For the cultures subjected to the seconds. 1 treatment with toxicant(s) the total amount of 2 [Ca<sup>2+</sup>]<sub>c</sub> was calculated for the same period of 250 3 seconds starting from the maximum amplitude. 4 recovery time of the control cultures was therefore: 5 6 total amount of [Ca2+]c (µM) for the toxicant-treated 7 samples over 250 seconds x 100 8 total amount of [Ca2+] (µM) for the control sample 9 over 250 seconds 10 11 ; and 12 13 Percentage Increase in pre-Stimulating [Ca2+]c 14 Resting Level, the percentage increase in [Ca2+]c 15 prior to the stimulus, where the control value is 16 taken to be 100%. 17 18 There is also the possibility of combining one or 19 more of these parameters to obtain further values 20 which can be used for identification of the 21 toxicants in the mixture. For example, the 22 summation of amplitude and recovery time will give 23 the value of total cytosolic free calcium ions 24 emitted from the time when [Ca2+] c reaches its peak. 25 · Also summation of lag time and rise time will give 26 the total time required for [Ca2+] c to reach its 27 peak. The division of final [Ca<sup>2+</sup>]<sub>c</sub> resting level 28 onto the pre-stimulation [Ca2+] resting level will 29 show how many times the [Ca2+]c resting level has 30 changed after stimulation. Similarly, a division of 31 the final [Ca2+] c resting level onto the initial 32

1	[Ca <sup>2+</sup> ] <sub>c</sub> resting level prior to the addition of
2	toxicant(s) gives further identifying data.
3	Additionally, the summation of all the data points
4	of the trace gives the total amount of cytosolic
5	free calcium ions released during the monitoring
6	period.
7	
8	The present invention will now be described with
9	reference to the following non-limiting examples and
10	with reference to the figures, wherein:
11	
12	Figure 1 shows the characteristic $[Ca^{2+}]_c$ trace
13	produced on addition of 5mM external CaCl2,
14	following a 5 minute pre-incubation with
15	different concentrations of 3,5-DCP.
16	
17	Figure 2 shows the caracteristic [Ca2+] c trace
18	produced on addition of 5mM external CaCl2,
19	following a 5 minute pre-incubation with
20	different concentrations of Cr6+.
21	
22	Figure 3 shows the characteristic $[Ca^{2+}]_c$ trace
23	produced on addition of 5mM external CaCl2,
24	following a 5 minute pre-incubation with
25	different concentrations of Zn2+.
26	
27	Figure 4 shows the characteristic $[Ca^{2+}]_c$ trace
28	produced on addition of 5mM external CaCl2,
29	following a 30 minute pre-incubation with
30	different concentrations of 3,5-DCP.

Figure 5 shows the characteristic [Ca2+] c trace 1 produced on addition of 5mM external CaCl2, 2 following a 30 minute pre-incubation with 3 different concentrations of Cr6+. 4 5 Figure 6 shows the characteristic [Ca2+] c trace 6 produced on addition of 5mM external CaCl2, 7 following a 30 minute pre-incubation with 8 different concentrations of Zn2+. 9 10 Figure 7 shows the characteristic cytosolic free 11 calcium ion concentration, [Ca2+]c, trace 12 produced on addition of 5mM CaCl<sub>2</sub> following a 5 13 minute pre-incubation with different 14 concentrations of 3,5-dichlorophenol, 3,5-DCP. 15 16 Figure 8 shows the characteristic [Ca2+] c trace 17 produced on addition of 5mM CaCl2, following a 18 30 minute pre-incubation with different 19 concentrations of 3,5-DCP. 20 21 Figure 9 shows the characteristic [Ca2+] c trace 22 produced on addition of 5mM CaCl2, following a 5 23 minute pre-incubation with different 24 concentrations of chromium ions, Cr6+. 25 26 Figure 10 shows the characteristic [Ca2+] c trace 27 produced on addition of 5mM CaCl2, following a 28 30 minute pre-incubation with different 29 concentrations of chromium ions, Cr6+. 30

Figure 11 shows the characteristic [Ca2+] c trace 1 produced on addition of 5mM CaCl2, following a 5 2 minute pre-incubation with different 3 concentrations of zinc ions, Zn2+. 4 5 Figure 12 shows the characteristic [Ca2+] c trace 6 produced on addition of 5mM CaCl2, following a 7 30 minute pre-incubation with different 8 concentrations of zinc ions, Zn2+. 9 10 Figure 13 shows the values of [Ca2+] c trace 11 parameters characteristic for different 12 concentrations of pentochlorophenol, PCP; sodium 13 dodecyl sulphate, SDS; and Toluene. Parameters 14 assessed are Lag Time, LT; Rise Time, RT; 15 Amplitude, A; Full Width Half Maximum, FWHM; 16 Percentage Increase in pre-Stimulating [Ca2+]c 17 Resting Level, %IpreSRL; Percentage Increase in 18 Final [Ca<sup>2+</sup>]<sub>c</sub> Resting Level, %IFRL; Percentage 19 Increase in Recovery Time, %IRT; and Number of 20 [Ca<sup>2+</sup>]<sub>c</sub> Increases. 21 22 Figure 14 shows the values of [Ca2+] c trace 23 parameters characteristic for 3,5-DCP, PCP, Zn2+, 24 Cr6+, Toluene, and SDS. Parameters assessed are 25 Lag Time, LT; Rise Time, RT; Amplitude, A; Full 26 Width Half Maximum, FWHM; Percentage Increase in 27 pre-Stimulating [Ca<sup>2+</sup>]<sub>c</sub> Resting Level, %IpreSRL; 28 Percentage Increase in Final [Ca2+] Resting 29 Level, %IFRL; Percentage Increase in Recovery 30 Time, %IRT; and Number of [Ca2+]c Increases. 31

Figure 15 shows the values of [Ca2+] c trace 1 parameters characteristic for different mixtures 2 Parameters assessed are Lag Time, of toxicants. 3 LT; Rise Time, RT; Amplitude, A; Full Width Half 4 Maximum, FWHM; Percentage Increase in pre-5 Stimulating [Ca<sup>2+</sup>]<sub>c</sub> Resting Level, %IpreSRL; 6 Percentage Increase in Final [Ca2+] Resting 7 ' Level, %IFRL; Percentage Increase in Recovery 8 Time, %IRT; and Number of [Ca2+] c Increases. 9 10 Effect of pre-incubation of Aspergillus awamori with 11 toxicants on [Ca2+] c response to external calcium 12 13 chloride 14 12 ml of sterile VS medium was inoculated with 1 x 15 10<sup>5</sup> spores per ml A. awamori strain 66A. 100 µl of 16 the inoculated medium was added to each well of a 17 96-well plate and cultured in a humidity chamber in 18 the presence of free water at 30 °C for 24 hours. 19 20 The following toxicants were tested: 3,5-21 dichlorophenol, zinc sulphate, and potassium 22 dichromate. Each toxicant was added in a total 23 volume of 25 µl VS medium or water 5 or 30 minutes 24 before addition of 5 mM calcium chloride. 25 26 Luminescence was monitored for 5 minutes following 27 addition of CaCl2. Aequorin was completely 28 discharged by adding 3M calcium chloride in 20% 29 ethanol. The total concentration is thus 1.5 M 30 calcium chloride in 10% ethanol. 31

Luminometry was performed using an EG & G Berthold 1 2 (Bad Wildbad, Germany) LB96P Microlumat luminometer. 3 Luminescence data was converted from real light units to  $[Ca^{2+}]_c$  values using the following equation: 4 5 6 PCa = 0.332588 (-log k) + 5.5593,7 where k = luminescence counts per second/total 8 9 luminescence counts. Total luminescence is measured as an integral of all luminescence up to complete 10 11 aequorin discharge. 12 The following parameters were assessed: 13 Rise Time, Amplitude, Full Width Half Maximum and 14 15 Final [Ca<sup>2+</sup>]<sub>c</sub> Resting Level. 16 Effects of different concentrations of toxicants on 17 [Ca<sup>2+</sup>]<sub>c</sub> traces 18 19 Aspergillus awamori were transformed with an 20 expression vector (pAEQ1-15) comprising a gene for 21 22 synthetic apoaequorin (aeqS) under the control of the constitutive glucose-6-phosphate dehydrogenase 23 24 promoter (gpdA). 25 26 These transformants were cultured in 100 µl of Vogel's medium with 1% sucrose (VS medium) in 27 microwell plates for 24 hours before addition of a 28 toxicant or a control of distilled water. 29 were dissolved in water to give the concentrations 30 shown below. 25 µl of the each of the following 31 32 concentrations were added to each culture:

CONCENTRATIONS (mg/1)
0.112, 11.2, 112
15, 120, 260
180, 350, 700, 1300

2

3 The cultures were incubated for 5 or 30 minutes

4 before addition of 100 μl 5mM CaCl<sub>2</sub>. Luminescence

5 was measured for 5 minutes using a plate

6 luminometer. Luminescence data was manually

7 converted from relative light units to cytosolic

8 free calcium ion concentration, [Ca2+]c. This was

9 then plotted against time and parameters of this

10 trace were analysed. Parameters assessed were as

11 follows:

12

13

Rise Time, the time from addition of CaCl2 to the

14 moment when maximum [Ca<sup>2+</sup>]<sub>c</sub> was achieved;

15 Amplitude, the maximum [Ca2+] c reached during the

16 experiment;

17 Full Width Half Maximum, the width of the transient

18 at the point where the amplitude equals half of the

19 maximum amplitude of the transient;

20 and Final Resting  $[Ca^{2+}]_c$  Level, the resting  $[Ca^{2+}]_c$ 

21 at the end of the experiment.

22 23

# Effects of further toxicants on [Ca2+]c traces

24

25 Cultures of Aspergillus awamori as described above

were used to test the effects of further toxicants.

27 The concentrations of toxicants tested were made up

as follows in water, where the concentrations tested were based on Dutch target and intervention values for toxicants and Kelly Guidelines for the classification of contaminated soils:

TOXICANT	CONCENTRATION (mg/l)
Pentochlorophenol, PCP	0.01, 0.1, 1, 5, 10
Sodium dodecyl sulphate,	1, 10, 50, 100, 500
SDS	
Toluene	1, 25
3,5-DCP	10
Zn <sup>2+</sup>	700
Cr <sup>6+</sup>	15

 In the first set-up (S1), 100 µl of each toxicant concentration or of the control (VS medium) were added to the cultures through built-in injectors and luminescence monitored for 5 minutes. In a second set of experiments (S2), cultures were pre-incubated with the toxicant or control for 5 minutes before addition of 5mM CaCl<sub>2</sub> in a total volume of 25 µl distilled water. Luminescence was monitored for 5 minutes following addition of CaCl<sub>2</sub>. Luminescence data was converted from relative light units to [Ca<sup>2+</sup>]<sub>c</sub> values as described above. The following parameters were assessed in S1:

- 20 Lag Time, the time from addition of CaCl<sub>2</sub> to the
- 21 time when [Ca<sup>2+</sup>]<sub>c</sub> began to rise;
- 22 Rise Time;
- 23 Amplitude;
- 24 Full Width Half Maximum;

```
Percentage Increase in Final [Ca2+] c Resting Level,
1
      where the control value was taken to be 100%;
2
      Percentage Increase in Recovery Time, where the
3
      control value was taken to be 100%;
4
      and Number of [Ca^{2+}]_c Increases, the number of [Ca^{2+}]_c
5
      transients.
6
7
      In S2, the Percentage Increase in pre-Stimulating
8
       [Ca^{2+}]_c Resting Level, where the control value was
9
      taken to be 100%, was assessed in addition to all of
10
      the parameters tested in S1.
11
12
       Effects of mixtures containing different proportions
13
       of toxicants on [Ca2+] c traces
14
15
       The experiments described when examining the effects
16
       of further toxicants were repeated for different
17
       mixtures of toxicants. The following mixtures were
18
       made up in water for testing:
19
20
       6 \text{ mg/l } 3,5\text{-DCP} + 12 \text{ mg/l } \text{Cr}^{6+}
21
       30 \text{ mg/l } \text{Cr}^{6+} + 350 \text{ mg/l } \text{Zn}^{2+}
22
       10 mg/l 3,5-DCP + 350 mg/l Zn^{2+}
23
       6 \text{ mg/l } 3,5\text{-DCP} + 12 \text{ mg/l } \text{Cr}^{6+} + 350 \text{ mg/l } \text{Zn}^{2+}
 24
                         20 mg/l Cadmium
        Mixture 1:
 25
                         100 mg/l Copper
 26
                          50 mg/l Chromium
 27
                          250 mg/l Zinc
 28
                          500 mg/l SDS
 29
                          20 mg/l Cadmium
        Mixture 2:
 30
                          100 mg/l Copper
 31
                          50 mg/l Chromium
 32
```

#### 1 250 mg/l Zinc 2 These experiments demonstrate a novel finding that 3 each toxicant results in a different and 4 characteristic [Ca2+] c transient. Additionally each 5 concentration of toxicant produces a unique [Ca2+]c transient. From these characteristic fingerprint 7 responses a profile of data can be built up and used 8 to create a bank of data for each toxicant. Results 9 from testing samples can be compared with this data 10 bank and the presence of a particular toxicant can 11 thus be determined. Furthermore, details such as 12 the mode of action of the toxicant, and the amount 13 of toxicant present can be deduced from a comparison 14 with the bank of pre-gathered data. 15 16 Although the invention has been particularly shown 17 and described with reference to particular examples, 18 it will be understood by those skilled in the art 19 that various changes in the form and details may be 20 made therein without departing from the scope of the 21

present invention.

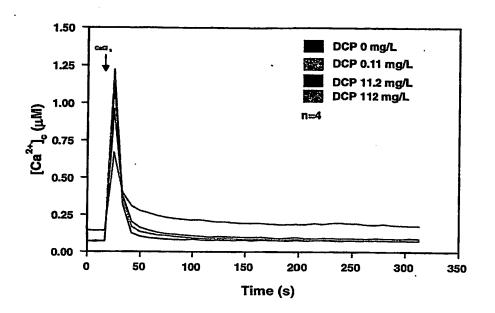


Figure 1

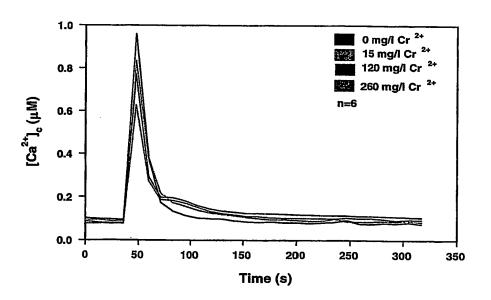


Figure 2

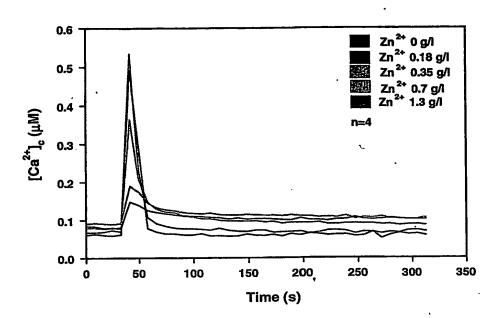


Figure 3

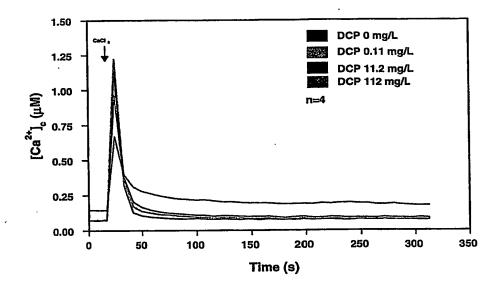


Figure 4



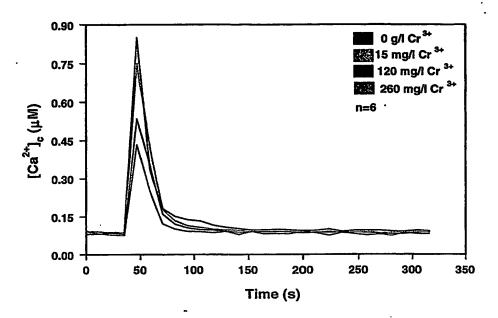


Figure 5

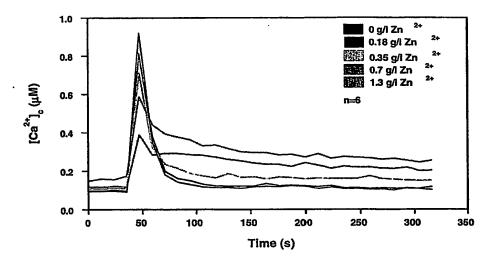


Figure 6

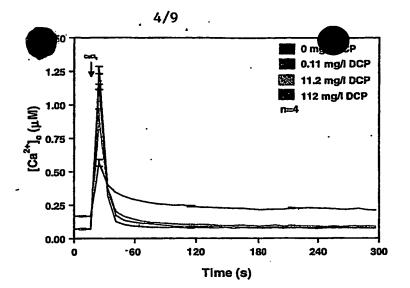


Figure 7

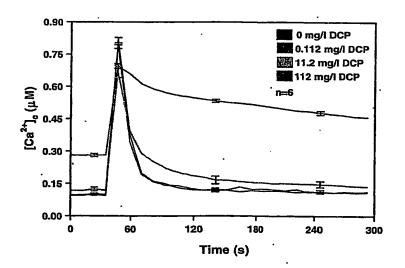


Figure 8

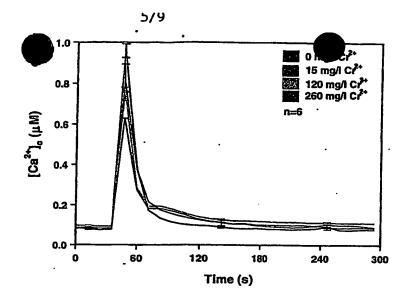


Figure 9

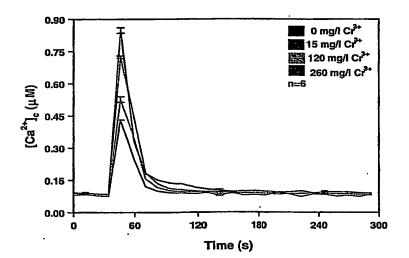


Figure 10

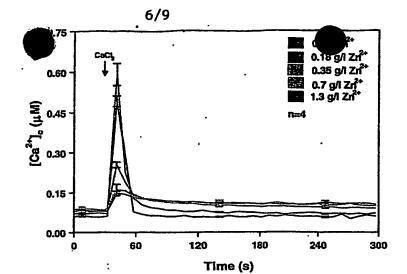


Figure 11

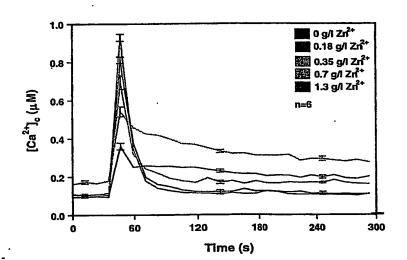


Figure 12

`)																
	Number of increases		<b>S2</b>	-	<del>-</del>		٠ .	<b>~</b>	-	-	۰ -	<b>-</b>	۰, ۲	<b>-</b>	п.а.	-
	Nur O incre		<b>S1</b>		-	· <del>-</del>		<b>-</b>	-	-	· -	, <u>.</u>	<b>-</b>	→ ,	<b>-</b>	n.a.
	%IRT		<b>S</b> 2	102±3	6 <del>76</del> 6	1001	21017	/T017	308±4	10919	154+10	287+10	207710	71±007	 g.	115±2
	[%		SI	111±4	122±9	195+8	274+4	1 1 1	373±22	114±6	243±14	328+6	367+11	20/111	203±12	п.а.
	%IFRL		<b>S</b> 2	104±6	104±11	107±11	205+9		723±7	106±7	120±13	222±16	256+14	0 5	# 5	113#3
		į	- N	115±6	11845	204±13	209±7	206	/ TECOC	11645	162±7	237±9	405±15	561+12		п.а.
	%IpreSRL	દ	70	6 <del>7</del> £6	104±9	10945	120±3	12140	<u> </u>	125±34	136±13	323±19	359±24	11.2	12010	12313
	FWHIM	S	70	•	,	1	<b>←</b>	<b>←</b>	_	,	<b>←</b>	<b>←</b>	<b>←</b>	n.a.		
		5	5 •	<del>-</del>	<b>←</b>	<del>(</del>	<b>←</b>	<b>←</b>	<b>-</b> .	1	<b>←</b>	<b>←</b>	<b>←</b>	<b>←</b>	æ	
	Ą		3	100±5	91±5	80±10	89±14	175+27		102±8	118±4	115±12	116±3	n.a.	74+13	
		SI		75±23	74±22	72±14	199±26	417±73		111148	246±19	295±33	293±19	998±35	n.a.	
	RT	SS	•	<b>→</b>	<b>-</b>	-	-	12.6	•	-	-	-	-	n.a.	,1	
		SI	-	<b>-</b>	<b>-</b>		12.6	12.6	•	<b>-</b>	12.6	12.6	12.6	12.6	n.a.	
	LT	S2	c	> (	0	0	0	0	<	>	0	0	0	n.a.	0	
	Н	S1	_	> (	0	0	0	0	_	>	0	0	0	0	n.a.	
Values	of interest (mg/l)		0.01	5 ,	<b>1.</b> 0	<b>.</b>	2	10	. —	- (	01	20	100	200	-	
	Chemical			•	<u> </u>	ටු						SDS			Toluene	

Note: LT= lag time
RT=rise time
A=changes in A (%)
FWHM=full width half maximum

%IpreSRL= % increase in pre-stimulation resting level %IFRL=% increase in final resting level %IRT=% increase in recovery time S1=Stage 1 S2=Stage 2

Figure 13

	Number of increases		82		_	-	<b>-</b>	-		, -	<b>—</b>	-
	Nu		SI	¦ •	-	_	<b>-</b>	_	-	٠ ٠	_	-
	%IRT		S2	00.00	70/±73	308+4	1	225±12	120428	071071	115±2	286±12
	%		SI	152112	CIECT	253±7   373±22		258±5	103+2		ж <u>і</u>	565±12
	%IFRL		S2	17777		253±7		263±14	110±11	11213		256±14   565±12
	1%		SI	134+10		305±17		7.75±8	104±5	a E	į	561±12
	%IpreSRL	8	22	102±3		13179	140110	142±18	102±12	121+6		359±24
	FWHM	5	70 10	<del></del>	+	_	+		•	<b>←</b>	•	
	FW	3	To _	<b>←</b>	+	_	<b>←</b>	-	•	n.a.	+	-
	· ¥	3	70	79±25	176107	1/3E/1	74+1	1	84±23	73±13	11/10	110±3
		5	5	- 40式	417472	C/T/14	42+5	]	41±5	n.a.	2000	770133
	L	8	}		126	777	-	,		₩,	_	-
	RT	153	,	12.6	12.6		12.6	•	<b>-</b>	n.a.	0 126	2
	LT	S1 S2	,	<b>-</b>	C	•	0	(	<b>&gt;</b>	0	C	,
		S1		<u> </u>	0	·	0		>	0	0	, ]
Value	of interest (mg/l)		5	07.	10	•	700	4	CT	25(1)	200	].
	Chemical		מיט ער איני	JOH C.C.	PCP	; -	Znz	ţ,	ゔ	Toluene	SDS	

Note: LT= lag time
RT=rise time
A=changes in A (%)
FWHM=full width half maximum

%IpreSRL= % increase in pre-stimulation resting level %IFRL=% increase in final resting level %RT=% increase in recovery time S1=Stage 1 S2=Stage 2

Figure 14

Number	increases		70		•	٠,	-	<b>-</b>	· -	<b>⊣</b>	r	۸ ،	7	7		2	۰ -	4
Ž	Ĭ.	ē	<u></u>	_	_	•	<b>-</b>	_	•	i.	_	• •	7	7		6	-	•
%RT		S	70.	207±23	120+28	27.750	71±c77	286±12	110+10	017711	154+6	208+11	117007	195±8		477±28	120+7	
%		15.		153±13	103±2	24026	4.00.	565±12	128+5		158426	311+14		16045		446±17	170±28	
%IFRL		SS.		1/17/1	110±11	263+14	1100	230±14	120±14		150±12	153±16		150±9		501±38	132±27	
<u></u>		SI	124410	017451	104±5	225+8	561±10	711100	117±4	•	143±18	294±18		164拉		402±17	177±44	
%IpreSRL		S2	100+3	1001	102±12	142土18	350+07	477600	96±26		100±5	103±9		102±8	,	262±13	148±16	
FWHIM	- 1	SI SZ	<b>+</b>	•	ı	<b>←</b>	<b>←</b>	-	, ←	•		· +	•	<u> </u>	<del>-</del>		l	_
A	5	78	79+25		84±23	74±1	116+3		88±13		/6±4	86±2		79±5	,	0 <u>T</u> 871	69±14	_
	3	Z	40+2	1	41±5	42±5	998±35		33±4	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	0.T.C.7	65±5	1	25±2	466.110	400±13	116±8	
RT	S	70	-	•	<b>-</b>	-	-		-	-	 <b>⊣</b>	-	•		10 6.	5		
L P¥	5	ī	12.6	-	<b>⊣</b>	12.6	12.6		12.6	1	7	12.6	,	17.0	19.6	2	<b>—</b>	
LT	S	30	0	<	>	0	0		0	_	>	0		<u> </u>			0	1
	5	5	0	_	>	0	0		0	_	٥	0	<	>	<b>C</b>	•	0	
Values of interest (mg/l)			.10	15	3	700	200		6+12	30+350		10+350	6+12+350	00042170	See	M&M	See M&M	1
Chemical			3,5 DCP	<del>ئ</del> ئ	; 1		SDS	3.5-DCP	+ C +	$Cr^{6+}Z_n^{2+}$		3,5DCP +Zn <sup>2+</sup>	3,5-DCP + 7-6+	$Z_n^{2+}$	Mixture .	⊣i	Mixture 2	

Italics represents data obtained with very high concentrations of toxicants: Zn<sup>2+</sup>=700 mg/l; Cr<sup>6+</sup>=120 mg/l; 3,5 DCP=49 mg/l

Figure 15

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